Demographic Differences by Age, BMI, Gender and Disease States of Phase I and Phase II Enzyme Activities in Cryopreserved Human Hepatocytes

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ABSTRACT

Human hepatocytes are a key in vitro reagent for making predictions of in vivo drug metabolism, interactions and intrinsic clearance in drug discovery and development. However, inter- individual differences in drug metabolizing enzyme activities complicate pharmacokinetics, leading to varying efficacy and drug-drug interactions. To delineate the potential influences, we have reviewed phase I (CYP1A2, 2A6, 2C9, 2C19, 2E1 and 3A4) and phase II (UGT and SULT) enzymatic activities as they relate to age, BMI, gender and ethnicity. The data was generated using cryosuspension hepatocytes with specific substrates (CYP1A2: phenacetin, 2A6: coumarin, 2C9: tolbutamide, 2C19: mephenytoin, 2E1:chlorzoxazone, 3A4:testosterone, UGT:7-hydroxycoumarin and SULT:7-hydroxycoumarin) near Km concentrations, as well as with multiple enzyme substrate 7-ethoxycoumarin (ECOD). From a minimum of 180 donors, several statistically significant trends were observed. For age-dependent differences, a loss of activity was observed for ECOD and CYP2C19, and an increase in CYP1A2 activity as the age increased from 1 to 89 years old. As BMI increased, ECOD, CYP1A2 and CYP2C19 decreased between the range of 14 and 53. As for gender-related differences, men showed higher activities in ECOD and CYP2E1. Diabetic donors had lower CYP2C9, CYP2C19 and CYP3A4 activities compared to non-diabetics. Overall, choosing appropriate hepatocyte preparations for metabolism studies as a reflection of "average" are dependent upon age, BMI and disease states in many drug metabolizing enzymes.
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Background

There are significant differences between individuals in enzymatic activities that affect the pharmacokinetics (PK) of drugs. These differences may be attributed to genetic dispositions, environmental influences or developmental parameters. Understanding the individual variations aids in the interpretation of how a drug will perform in the general population.

Several key factors have been observed in clinical setting. For example, Type I diabetic patients have been shown to metabolize antipyrine and caffeine more than healthy volunteers indicating higher CYP1A2 activity.[1] In another study, Type II diabetic patients showed an increased activity of CYP2E1 via chlorzoxazone metabolism as compared to healthy participants and Type I diabetic patients. This suggests a differential expression between healthy and diabetic patients and between Type I and Type II diabetics.

Ethnicity is another critical delineation of the population especially in heterogeneous societies such as the United States of America. One substantial influence in ethnic differences is polymorphisms of drug metabolizing enzymes. For example, 5-14% of Caucasians are CYP2D6 poor metabolizers, while only 0-5% of Africans and 0-1% of Asians are CYP2D6 poor metabolizers.[3] In the case of warfarin, individuals with CYP2C9*1/*2 required 20% lower dose than wild-type individuals to maintain sufficient anticoagulant therapy. A genetic survey revealed that Asians do not have *2 polymorphism, while 2.9% of Blacks and 10% of American Caucasians do.[4]

Age may be a significant factor to PK. A review of caffeine, midazolam, morphine and paracetamol found significantly reduced metabolism in patients under two years old, while median plasma clearance values of those from two years old and older were similar.[5] Other factors beside enzymatic activity may influence drug clearance as well. For example, age-related blood flow differences have been suggested as a reason for altered intrinsic clearance.[6]

Obesity is a prevalent concern for clinicians and pharmaceutical scientists. A review of clinical studies comparing obese and non-obese patients has implicated drug metabolism, liver blood flow, glomerular filtration and tubular processes as key influences. CYP3A4 was reported to be lower in obese patients, while UGT and CYP2E1 are higher.[7]

Herein, we surveyed phase I and phase II enzymatic activities from cryopreserved human hepatocytes for trends in respect to age, body mass index (BMI), ethnicity and diabetes in the context cellular function which is devoid of distribution, blood flow and other whole-body parameters.

Materials and Methods

Enzyme Characterization: Cryopreserved human hepatocytes were stored at < -150° C prior to enzyme characterization. Three vials from each donor were used to determine the enzymatic activities of Phase I and Phase II drug metabolizing enzymes as measured by metabolite formation. In Table 1, the enzymes, specific substrates, reaction final concentrations, and their associated metabolites are listed. Cryopreserved hepatocytes were thawed in 37° C water bath for approximately two minutes then transferred into 48 mL of InVitroGro HT medium at 37° C. Cell suspension was centrifuged at 50 x g for five minutes at ambient temperatures. The supernatant was removed and the cell pellet was resuspended in two mL of InVitroGro KHB. The cell viability and concentration was determined by Trypan blue exclusion. The cell suspension was diluted to $2x10^6$ viable cells per mL. Substrates were diluted to 2X final in InVitroGro KHB. The cell suspension and 2X substrates solutions were incubated for 5 minutes at 37 °C. Reaction was initiate by adding equal volumes (100 µL) of cell suspension and substrate solutions in 48-well culture plate and incubated for one hour in 37° C humidified 5% CO_2 incubator. Reactions were stopped with equal volume methanol. Reaction samples were transferred to cryovials and stored at -80° C prior to bioanalytical analysis. Substrate and metabolite concentrations were measured by HPLC and UPLC/MS/MS methods.

Enzyme	Substrate	[µM]	Metabolite(s)
CYP1A2	Phenacetin	15	Acetaminophen
CYP2A6	Coumarin	8	7-HC, 7-HCG, 7-HCS
CYP2C9	Tolbutamide	150	4-OH Tolbutamide
CYP2C19	S-mephenytoin	20	4-OH Mephenytoin
CYP2D6	Dextromephorphan	8	Dextrorphan
CYP2E1	Chlorzoxazone	100	6-OH Chlorzoxazone
CYP3A4	Testosterone	50	6β-OH Testosterone
ECOD	7-ethoxycoumarin	75	7-HC, 7-HCG, 7-HCS
SULT	7-hydroxycoumarin	30	7-HC Sulfate
UGT	7-hydroxycoumarin	30	7-HC Glucuronide

Table 1. List of substrates, reaction concentrations and specific metabolites for the associated phase I and phase II metabolic enzymes.

Calculations: The enzymatic rates were derived from the concentrations of the metabolites formed per reaction and normalized to cell number and incubation time, and expressed in pmoles of metabolite per minute per million hepatocytes ($pmol/min/10^6$).

Statistical Analysis: A database was generated with enzymatic rates and donor demographic data (age, ethnicity, BMI and gender). Age and BMI effects on enzyme activities were analyzed by linear regression and its significance from non-zero slope. Diabetic influences on enzymatic activities were compared by t-test and 1 way ANOVA. Ethnicity (Caucasian, African American and Hispanic) influence on enzyme activities were compared by 1 way ANOVA. All statistical analysis and graphs were generated using GraphPad Prism 5 software.

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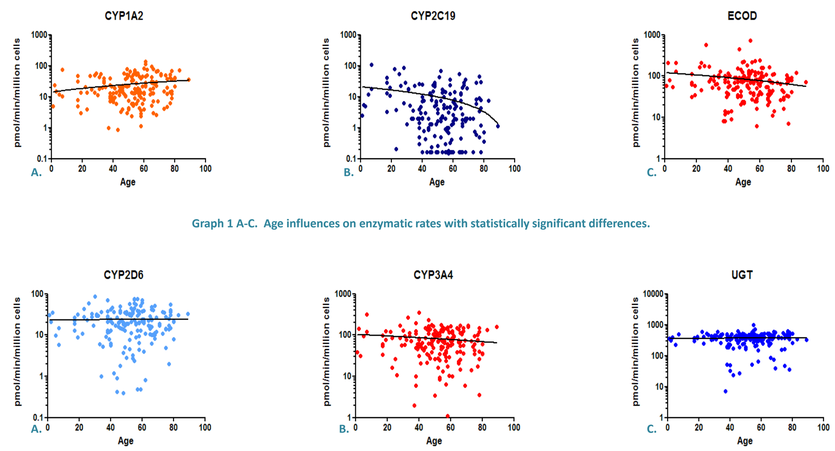
AGE

Results: Age of the donor was plotted with respect to individual phase I and phase II enzymatic activities. The age ranged from 1 to 89 years old with the mean and median age of 51 and 53, respectively. CYP2A6, 2C9, 2D6, 2E1, 3A4, UGTs and STs showed no statistical significance from non-zero slope. CYP3A4 does appear to trend towards lower activity with an increase in age but achieved p-value of 0.09 (Graph 2B). With a larger data set, this trend may be confirmed to be significant.

CYP1A2 showed a marked increase in the activity with an increase in age with a p-value of 0.023. CYP2C19 activity decreased as age of the donor increased, p-value 0.001. ECOD activity which is a measure of phase I and phase II activities, decreased as the donor's age increased with a p-value of 0.038.

Discussion and Conclusion: ECOD is a broad-based predictor of both phase I and phase II metabolism. Ethoxycoumarin (7-EC) has been reported to be metabolized by CYP1A1 and 2E1 predominantly with minor metabolism by CYP1A2, 2A6, 2B6, 2C8 and 3A4 to form 7-hydroxycoumarin (7-HC)[8], and subsequent conjugation by UGTs and STs. ECOD reduction of broad metabolism by age may be a reflection of general metabolic capacity. This trend has been reported by other broad-based substrates, such as antipyrine.[11, 12] Loss of CYP2C19 activity in microsomes and hepatocytes has been reported[13] and observed in clinical setting.[14] CYP1A2 delayed ontology has been reported, though the comparison of the age cohorts were between fetal, new-born up to 10 years old and adult and not a delineated adult age response.[15] CYP3A4 reduction due to age has not been reported[16] though a slight negative trend is observed in this data set.

Age may affect the metabolism of a drug depending on its reaction phenotyping. Other influences like BMI and gender may need to be stratified to better assess age-related correlation to activities.



Graph 1 A-C. Age influences on enzymatic rates with statistically significant differences.

Graph 2 A-C. Representative graphs of age influences on enzymatic rates with no statistically significant differences.

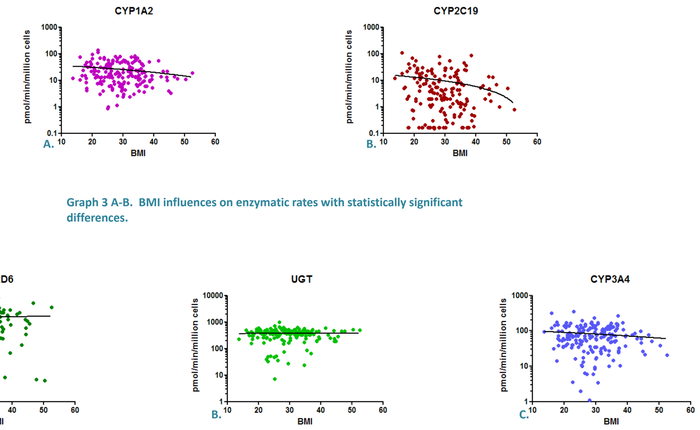
BMI

Results: BMI of the donor was plotted with respect to individual phase I and phase II enzymatic activities. The BMI ranged from 14 to 53 with a mean and median BMI of 29. BMI categories are underweight < 18.5, normal 18.5 - 24.9, overweight 25 - 29.9 and obese >30[9]. CYP2A6, 2C9, 2D6, 2E1, 3A4, ECOD, UGTs and STs showed no statistical significance from non-zero slope. CY3A4 does appear to trend towards lower activity with an increase in BMI but achieved p-value of 0.122 (Graph 4C). ECOD, too, trended lower without significance as BMI increased with a p-value of 0.071. With a larger data set, these trends may be confirmed to be significant.

CYP1A2 showed a marked decrease in the activity with an increase in BMI with a p-value of 0.020. CYP2C19 activity decreased as BMI of the donor increased, p-value 0.025.

Discussion and Conclusion: CYP1A2 inverse relationship with BMI has been reported in pre-menopausal women[17] but contradicts reported increase of clearance of drugs metabolized by CYP1A2 and 2C19 in the general population.[7] No citations were found linking BMI and CYP2C19 decreases. Polymorphisms of CYP2C19 will need to be segregated within BMI ranges to isolate BMI's influence on its activity.

Clinical observations of decreased CYP3A4 activity with an increase of BMI[7] was not confirmed in a statistically significant manner though a negative trend line was observed. The clinical observations may be due to CYP3A4 activity in other tissues such as the intestines as well as within the liver. More donors will be needed to confirm a connection between BMI and CYP3A4 activity in hepatocytes.



Graph 3 A-B. BMI influences on enzymatic rates with statistically significant differences.

Graph 4 A-C. Representative graphs of BMI influences on enzymatic rates with no significant differences.

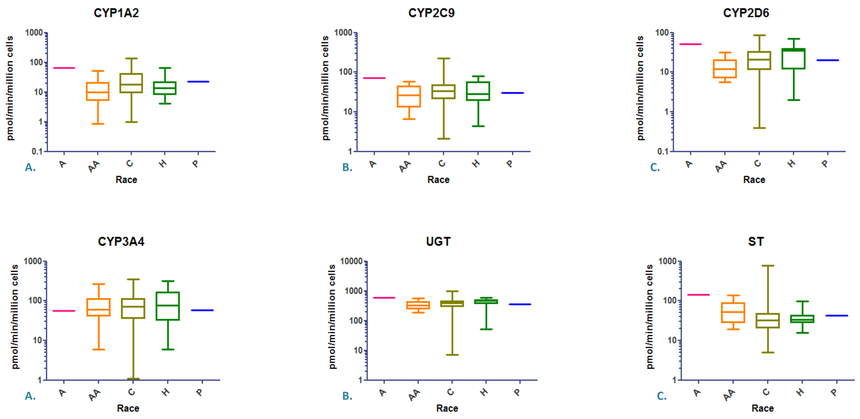
Ethnicity

Results: Ethnicity of the donors were compared with respect to grouped phase I and phase II enzymatic activities. The distribution of the donors reflected some bias from population of the United States of America. Caucasians represented 83% of the total number of donors compared to 2010 census data[10] of 72%. African Americans represented 7% of donors and 13% of the population. Hispanics represented 9% of the donors and 16% of the population. One donor of Asian and one donor of Polynesian decent were also included in the data set but were not used for statistical comparison given N=1.

No statistical significance was observed for any of the enzymatic characterizations performed between the three groups.

Discussion and Conclusion: Our survey found no correlation between ethnic groups for phase I and phase II enzymatic activities which does not agree with previously published works which described an increase of CYP2A6 and decrease of CYP1A2 in Hispanic donors as compared to Caucasians and African Americans.[13] But as noted by Parkinson, their findings may be an artifact of the low number of Hispanic donors in their data set.

Ethnicity may not affect drug metabolism and therefore selection of a donor group is not necessary as compared to the general public. Polymorphisms have not been compared and may have an influence on certain metabolic enzymes. More donors are necessary to assess under-represented groups such as Asians.



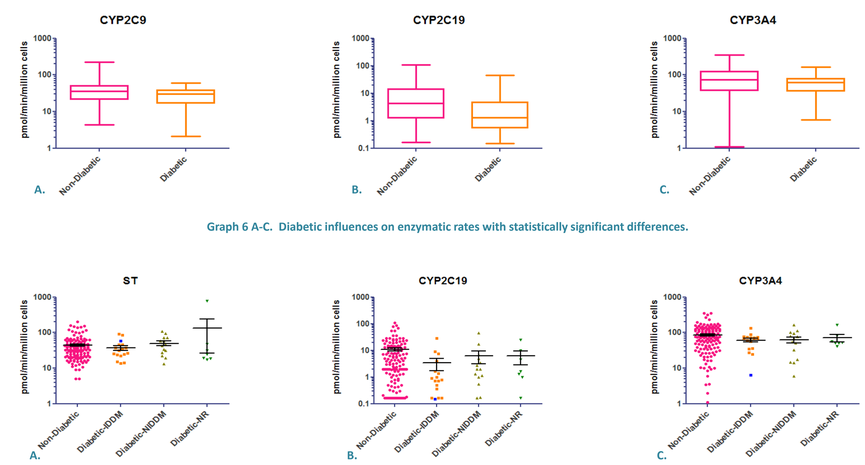
Graph 5 A-F. Representative graphs of Ethnicity influences on enzymatic rates with no significant differences.
A = Asian, AA = African American, C = Caucasian, H= Hispanic and P= Polynesian.

Diabetic Donors

Results: A comparison was performed between diabetic and non-diabetic donors using t-test. Statistically significantly lower activities were observed in diabetic donors for CYP2C9 (p-value 0.03), 2C19 (p-value 0.04) and 3A4 (p-value 0.04). There were 38 diabetic donors versus 131 non-diabetic donors. To further dissect the population, diabetic donors were segregated into insulin-dependent diabetes mellitus (IDMM) (N=17) which is also know as Type I, non-insulin-dependent diabetes mellitus (NIDMM) (N=14) which is also known as Type II and diabetic donors with type of diabetes not recorded (NR) (N=7). Further delineation such as Type II that has progressed to insulin-dependent stage could not be determined with information provided. One IDDM donor that was non-compliant with treatment has been plotted with blue dot.

Discussion and Conclusion: CYP2C9 importance have been reported in the clinic and are associated with SNP distribution.[18, 19] These have profound consequences with drug therapies such as sulfonylureas which are substrates for CYP2C9.[20] However, without genotyping, it is difficult to determine if the lower activity is due to diabetes or just a function of the polymorphism. No significance was observed when CYP2C9 was compared between non-Diabetics, IDDM, NIDDM and NR. CYP2C19 difference, too, may be a function of polymorphisms and not diabetic state. To complicate the matter, CYP2C19*2 has been noted as more prevalent in diabetics than with healthy volunteers in Mexico.[21] Similar genotypes of CYP2C19 do exhibit varying metabolism which may implicate a influence beyond polymorphism distribution.[22] Further investigation of polymorphic variance is warranted to explain CYP2C9 and CYP2C19 lower activity in diabetic donors.

Diabetes and polymorphism links have been reported for CYP3A4 in Japanese population.[23] However, functional differences have not been reported.



Graph 6 A-C. Diabetic influences on enzymatic rates with statistically significant differences.

Graph 7 A-C. Distribution of diabetic influences as delineated by non-diabetic, IDDM , NIDDM and not reported (NR) designations on enzymatic rates . ST and CYP2C19 were statistically significant, however CYP3A4 was not significant but included for comparative sake. Blue point in IDDM column represents a donor know as non-compliant for therapy.